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Natural autoantibodies, IgG antibodies to tetanus toxoid and CD5$^+$ B cells in patients with Mediterranean visceral leishmaniasis

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SUMMARY

Natural autoantibodies (NaAb) and IgG antibodies to tetanus toxoid (TT) were analysed in the sera of 38 children with active visceral leishmaniasis (VL) previously vaccinated with TT and in 30 healthy controls matched for sex and age. Patients exhibited high levels of NaAb to a panel of self antigens (tubulin, myosin, myoglobin, actin) contrasting to a low level of IgG to TT. Analysis of the circulating B cells in 26 untreated patients showed a low percentage of CD5$^+$ per total B cells (3–66%, mean 36·6%) compared with 14 normal controls (17·8–66·6%, mean 52·7%) ($P < 0.001$). Evaluation of these parameters after antimonial therapy showed a significant decrease of the level of the NaAb ($P < 0.0005$), and a spontaneous increase of the level of the IgG to TT without any vaccine boosting ($P < 0.01$). In contrast, there was a significant increase in CD5$^+$ B cells ($P < 0.0005$). This result suggests that CD5$^+$ B cells may be sequestrated in parasitized lymphoid organs and may be released after remission. These findings show that the polyclonal B cell activation that occurs during active VL involves mainly B cells bearing NaAb and are in favour of a functional dichotomy of B cells.

Keywords visceral leishmaniasis natural antibodies anti-tetanus toxoid antibodies B cell populations

INTRODUCTION

Autoreactive B cells producing natural autoantibodies (NaAb) account for a substantial part of the B cell repertoire [1–3], especially in the neonatal period [4–6]. These antibodies are constantly present in the sera of healthy humans [7], mice [2], rats [8] and fishes [9]. IgM with low intrinsic affinity to self and foreign antigens are the most abundant among NaAb immunoglobulins. In humans, some studies have shown that they can also be associated with IgG and IgA molecules [10]. The polyreactive IgM, IgG and IgA antibodies use preferential V genes in virtually unmutated configuration [11,12]. In mice, it has been reported that the ly-1b (CD5$^+$) B cell subpopulation, which are now called B-1 cells, makes autoantibodies [13]. The human homologue of B-1 cells displayed a similar repertoire to its murine counterpart. This B cell subpopulation is predominant at birth and represents about 1–5% of peripheral mononuclear cells in normal human adults [14]. At the clonal level, several studies have shown that natural polyreactive antibodies are produced by the CD5$^+$ B cells, whereas antigen-driven monoreactive antibodies are produced by conventional CD5$^-$ B cells ([15] for review). Regulation of these two B cell populations and the precise role of NaAb are still not clear. However, an increase of the titres of NaAb was reported in patients suffering various infectious and autoimmune diseases [16–19].

Visceral leishmaniasis (VL) is a parasitic disease caused by an obligate intracellular parasite *Leishmania infantum* [20]. During the acute phase, the host immune response to parasite is characterized by a T cell anergy to leishmanial antigens [21], contrasting with high levels of parasite-specific and non-specific antibodies, circulating immune complexes and rheumatoid factors [22]. High levels of polyreactive autoantibodies have been reported in VL [22,23].

In this study we investigated the autoreactive repertoire expressed by IgG antibodies against evolutionarily conserved molecules (tubulin, myosin, myoglobin and actin) compared with exogenous antigen-driven antibodies (IgG to tetanus toxoid (TT)). The percentage of CD5$^+$ B cells in patients with active VL was determined before and after treatment. Our data show that the polyclonal B cell activation that occurs in VL produces mainly autoreactive antibodies.

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PATIENTS AND METHODS

Patients

Thirty-eight children admitted in different paediatric departments of Tunis hospitals for characteristic symptoms of active VL were studied (19 females and 19 males; 1–7 years old, mean age 32 ± 12 months).

In all cases, the diagnosis of VL was established by serology and identification of parasites in bone marrow aspirates by using Giemsa staining or culturing on NNN medium. Twenty-eight patients were studied on admission, and 10 patients between day 1 and day 5 of antimonial therapy. Sixteen patients were studied before and after one course ($n = 10$) or two courses ($n = 6$) of antimonial therapy. Thirty healthy children (sex- and age-matched) were studied as controls.

All patients and controls had received three doses of DTP vaccine during the first year of life according to the Extended Program of Immunization as assessed by their vaccination card.

Cell preparation and staining

Mononuclear cells were isolated from heparinized peripheral blood by Ficoll–Hypaque gradient centrifugation and were resuspended in RPMI 1640 medium (Flow Labs, McLean, VA). Lymphocyte phenotyping was performed by flow cytometry analysis in an EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL). Staining was performed using predetermined optimal concentrations of unlabelled MoAbs including anti-CD3, anti-CD4, anti-CD8, anti-CD19, and as a second antibody an FITC goat anti-mouse immunoglobulin antibody (Immunotech, Marseille, France). Determination of $CD5^+$ B cells was performed by dual fluorescence using FITC-conjugated anti-CD20 and PE-conjugated anti-CD5 (Immunotech). Controls included either unstained cells or cells treated with an irrelevant unlabelled or FITC- or PE-conjugated MoAb.

Determination of IgG antibodies by enzyme immunoassay

Human muscle actin, calf muscle myosin and rabbit tubulin were a generous gift from Dr T. Ternynck (Institut Pasteur, Paris, France), whale skeletal muscle type II myoglobin was purchased from Sigma (St Louis, MO), and tetanus toxoid antigen from Institut Merieux (Lyon, France).

Screening of sera was done by enzyme immunoassay as follows. Polystyrene plates (CML, Nemours, France) were coated with a variety of antigens at final concentrations of 5 μg/ml for tubulin and 3 μg/ml for actin, myosin, myoglobin, and TT in 100 mM carbonate/bicarbonate buffer pH 9·6. The plates were incubated for 1 h at 37°C, then overnight at 4°C, and finally washed twice with PBS containing 0·1% Tween 20 (PBS-T). Unreacted sites were blocked by incubation with 0·5% gelatin in PBS-T (PBS-T-G) at 37°C for 1 h. Wells were washed and filled with 100 μl of serial dilutions of sera from VL patients and controls in PBS-T-G (1:100, 1:300, 1:900 for autoantibodies, and 1:500, 1:1000 and 1:2000 for TT antibodies). After 2 h incubation at 37°C, wells were washed and 100 μl of affinity-purified goat anti-human IgG (gamma specific) antibodies conjugated with peroxidase (Sigma) diluted 1:2000 in PBS-T-G were added to plates and incubated for 1 h at 37°C. After washing, 100 μl of orthophenylenediamine (0·7 mg/ml) with 0·03% hydrogen peroxide in citrate buffer (0·1 M, pH 5) were added. The colour development was measured after 15 min at 492 nm in a Titertek Multiskan (Flow Labs, Irvine, UK).

Estimation of total IgG level was made by standard radial immune diffusion technique (Mancini) using anti-IgG immune sera (Institut Pasteur, Tunis, Tunisia). Results were expressed in mg per 100 ml of sera.

Rheumatoid factor detection and absorption from test sera

Sera were investigated for the presence of rheumatoid factor (RF) by latex agglutination test (Arthrislidex; BioMerieux, Mercy l'Etoile, France). In order to study the effect of RF on autoantibodies and antibodies to TT titration, sera (diluted 1:10 in PBS) were depleted of RF by absorption (v/v) on IgG-coated latex particles (RF absorbant; Behringwerke AG, Marburg, Germany). After 2 h incubation at room temperature, the supernatant was collected by centrifugation and tested for RF reactivity and then for IgG anti-tubulin, anti-myosin, anti-actin and anti-TT as described.

Inhibition experiments

Patient sera were diluted 1:50 in PBS and preincubated (v/v) with inhibitors (actin, tubulin or myosin) at various concentrations (2, 20, 200 and 2000 μg/ml). After 2 h at 37°C, the reactivity of antibodies in each sample was determined by ELISA on actin-coated plates as described above. The results were expressed as the percentage of binding inhibition to plates compared with sample not incubated with inhibitor.

Expression of results

The values obtained by ELISA with a given serum sample and antigen were compared with the mean value for the same antigen obtained with 30 normal control sera examined under the same conditions. The results were expressed as the percentage of the OD (% OD) of the test serum compared with the mean value of the controls. The cut-off value for normal sera was defined as mean OD + 2 s.d. For IgG anti-TT, ODs obtained with a given serum sample were compared with those obtained with dilutions of specific human immunoglobulins (10 U/ml; Immunotetan, ISI, Napoli, Italy) and results are also expressed as U/ml. Protective titre to TT was defined according to WHO recommendations as 0·01 U/ml.

Statistical analysis

Student's one-tailed, two-tailed and paired *t*-tests were used for comparison between groups and between different samples for the same patients, with the level of significance set at 0·05. The distribution of OD for patients and controls being positively skewed, a log transformation was performed in order to allow the statistical tests.

RESULTS

Natural autoantibodies and IgG antibodies to TT

Sera from 38 patients with VL were tested by ELISA for the presence of IgG autoantibodies to self antigens: tubulin, myosin, myoglobin and muscle actin; and for IgG antibody to exogenous antigen, TT. Patients and controls had been previously vaccinated but not recently boosted with TT. Results were compared with those obtained with sera from 30 healthy controls (age- and sex-matched). Figure 1 shows that VL patients' sera had a significantly high level of IgG autoantibodies to all antigens tested compared with controls ($P < 0·0005$). IgG antibodies to self antigens were not equally

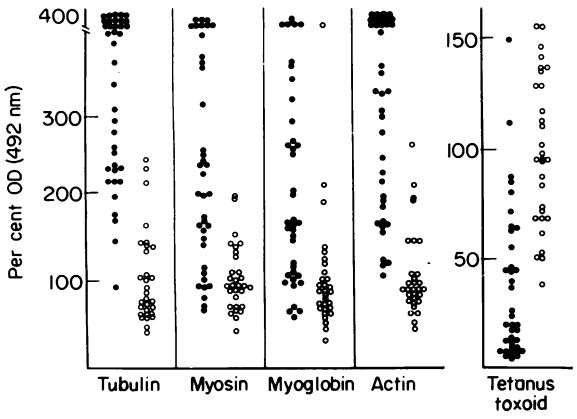

Fig. 1. IgG natural autoantibodies (NaAb) and IgG antibodies to tetanus toxoid (TT) in sera from 38 patients with visceral leishmaniasis (VL) (●) and 30 healthy controls (○). Results were expressed as a percentage of the mean OD obtained with 30 normal sera (100%).

enhanced, although a good correlation was found when autoantibodies were compared two by two ($R^2 > 0{\cdot}358$). IgG anti-tubulin antibodies were especially enhanced, while anti-actin antibodies were enhanced to a lesser extent. The percentage of sera positive for IgG anti-tubulin and IgG anti-actin antibodies were 86% and 68·4%, respectively (Table 1). In contrast, low levels of IgG anti-TT antibodies were found in the sera of VL patients compared with normals ($P < 0{\cdot}0005$). In spite of correct vaccination only 42% of patients had a protective titre of IgG to TT (0·01 U/ml) compared with 100% of controls.

High levels of total serum IgG were found in patients (1800 ± 1010 mg/100 ml (mean ± s.d.)), 80% of patients had total IgG levels higher than the upper limit of normal levels corrected for age (700–1000 mg/100 ml), and 30% of patients had a very high level of IgG (more than 2000 mg/100 ml). No correlation was found between levels of either total IgG, IgG autoantibodies, or IgG antibodies to TT.

RF were previously reported in VL [22,23]. In order to study if they interfered with the assays, sera were investigated for the presence of RF by latex agglutination test. RF were detected in 18/33 patients' sera (titre 1:20 to 1:640). Patients were separated into two groups according to the presence of RF. We found that VL patients with RF ($n = 18$) had significantly high levels of autoantibodies to all antigens tested compared with those without RF ($P < 0{\cdot}005$ for anti-myosin and anti-actin; $P < 0{\cdot}05$ for anti-tubulin and anti-myoglobin). However, as for the whole VL sera, the levels of autoantibodies of the 15 patients' sera without RF were also found to be significantly higher than those of control sera ($P < 0{\cdot}0005$ for anti-tubulin and anti-actin; $P < 0{\cdot}01$ for anti-myosin and anti-myoglobin). Furthermore, four sera with high NaAb and RF levels were comparatively tested before and after removal of RF. Although we found a marginal decrease of NaAb reactivity (range from 16% to 36%), the residual level of NaAb was still higher than that of control sera.

Finally, IgG to TT was found to be even lower in patients with RF compared with those without RF ($P < 0{\cdot}05$).

Table 1. Percentage of sera positive* for IgG to tubulin, myosin, myoglobin, actin and tetanus toxoid†

	Patients (%) ($n = 38$)	Controls (%) ($n = 30$)	P
Tubulin	86	10	$< 0{\cdot}0005$
Myosin	57·8	6·6	$< 0{\cdot}0005$
Myoglobin	39·4	3·3	$< 0{\cdot}0005$
Actin	68·4	6·6	$< 0{\cdot}0005$
Tetanus toxoid	42·1	100	$< 0{\cdot}0005$

* A sample was considered positive for autoantibodies when OD obtained by ELISA exceeded the mean + 2 s.d. values of control sera.

† Positivity was defined according to standard IgG assayed in the same conditions, and corresponds to 0·01 U of anti-tetanus toxoid antibodies per ml of serum.

Inhibitions experiments

In order to study the polyreactivity of NaAb found in the sera of patients with VL, autoantibodies were investigated by an inhibition competition assay with actin and other self antigens on actin-coated plates. Antigen-specific inhibition varied with the patient's serum being evaluated. Actin, tubulin and myosin effectively inhibited binding of IgG antibodies to actin (Fig. 2), whereas TT was ineffective (data not shown). On the other hand, preincubation of VL or normal sera with actin or tubulin or myosin at different concentrations had no significant inhibitory

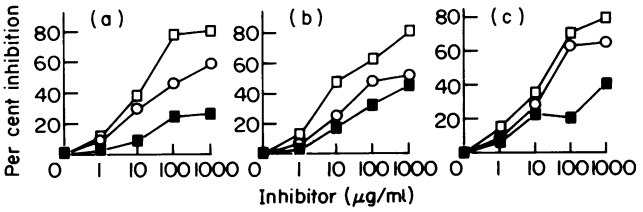

Fig. 2. Inhibition experiments. Three sera (a, b, c) with high anti-actin and anti-tubulin antibody titres were selected. After preincubation at a final dilution of 1:100 with increasing concentrations (1, 10, 100 and 1000 μg/ml) of actin (□), tubulin (○) or myosin (■) for 2 h at 37°C, sera were tested on actin-coated plates by ELISA. The results are expressed as percentage of inhibition.

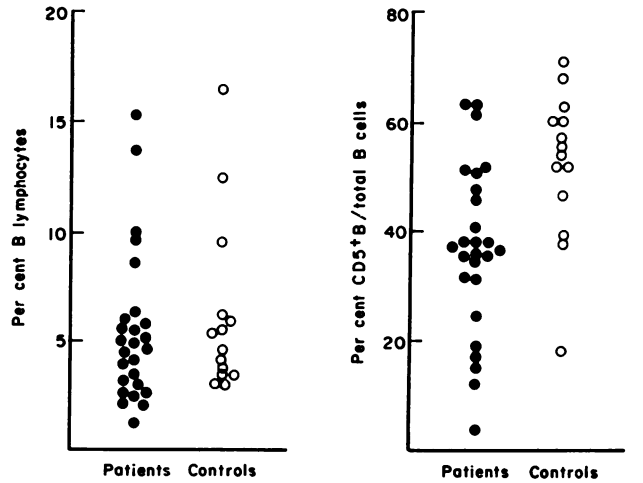

Fig. 3. Circulating total B ($CD20^+$) and $CD5^+$ B cells in patients with visceral leishmaniasis (VL) (●) and control subjects (○).

Table 2. Natural autoantibodies (NaAb), IgG antibody to tetanus toxoid (TT) and circulating B and CD5+ B cells in patients with visceral leishmaniasis analysed before and after therapy

Patient's age (months)/sex	Number of course, antimony	Time between two determinations (days)	Mean NaAb* (OD × 10^3)		IgG anti-TT* (OD × 10^3)		Total B cells (CD20+) (%)		CD5+ per total B cells (%)	
			Before	After	Before	After	Before	After	Before	After
36/M	1	150	850	869	708	659	5·5	12·1	63·6	65·8
20/M	1	30	403	394	1115	1235	3·4	1·8	17·6	33·3
36/M	2	30	861	765	1134	1342	4·9	5·3	24·4	39·6
17/F	1	40	569	365	451	952	4·3	3·7	37·2	51·3
16/M	1	20	397	310	233	374	9·7	2·4	18·5	33·3
74/F	1	20	1282	959	1014	1372	2·1	1·8	52·3	61·1
48/F	2	20	1182	934	1013	1092	3·1	5·8	64·5	65
24/F	1	60	890	565	1018	1134	1·1	4·9	63·3	65·3
36/M	1	60	418	314	642	503	2·2	5·2	36	82·6
13/F	1	20	1249	962	329	561	4·5	5·1	38·6	43
12/M	1†	20	2414	2006	249	300	5·9	1·1	15·2	26·6
24/M	1	120	888	633	468	623	6	10·6	31·1	66·6
30/F	2	60	480	360	1245	1400	3·9	5·7	15·3	40·3
13/M	2	20	1240	224	220	380	15·4	6·4	52·5	71·8
18/F	2	60	582	320	648	720	2·7	6	3	40
18/M	2	45	640	410	510	550	5·8	6	12·2	55

* NaAb and IgG antibody to TT were assayed for all patients before and after treatment on the same experiment using frozen sera.
† Patient died at day 30 after onset of treatment.

effect on the binding of IgG to TT-coated plates (data not shown).

Analysis of peripheral blood B cells

We have analysed the percentage of CD5+ B cells in peripheral blood mononuclear cells (PBMC) of 26 patients with active VL and 14 healthy controls (sex- and age-matched). The fraction of CD5+ B cells per total B cells ranged from 3 to 66 and 17·8 to 66·6 in patients and controls, respectively (Fig. 3). Interestingly, although there was no significant difference in the percentage of total B cells between patients and controls, a significant low percentage of CD5+ B cells was found in patients compared with normals ($P < 0{\cdot}001$). There was no correlation between the per cent of CD5+ B cells and the level of total IgG or IgG antibodies to tubulin, myosin, myoglobin, actin and TT.

Follow-up study

NaAb, IgG to TT, and circulating B and CD5+ B cells were further analysed in 16 patients before and after antimonial therapy. Serological assays were performed at the same time, using frozen sera, collected before and after treatment. NaAb and antibodies to TT were titrated by ELISA on the same plate coated in each case with a different antigen.

Ten patients were cured after one course of antimony treatment and were analysed at day 20–150 after initiation of treatment. Six patients needed two courses of antimonial drug and were further analysed during the second course ($n = 2$) and after the end of treatment ($n = 4$) between day 45 and day 60. Table 2 summarizes the clinical features and the results of the study.

The level of autoantibodies expressed as the mean of OD obtained by ELISA for IgG anti-tubulin, anti-myosin and anti-actin showed a significant decrease in 15/16 patients after treatment ($P < 0{\cdot}0005$). Interestingly, at the same time, a reciprocal significant spontaneous increase of IgG to TT was shown in 15 patients without any boost of TT ($P < 0{\cdot}01$). Although the percentage of circulating CD5+ B cells was variable (27–83%), a significant increase in all patients studied was noticed ($P < 0{\cdot}0005$).

DISCUSSION

The presence of autoreactive B cells is apparently not harmful to the organism. In fact, autoreactive B cells are supposed to play a role in the coordinated development of the early immune system [24–26] and to function as a primary defence mechanism [24,27,28]. NaAb are found in normal human sera [1] and supernatant of Epstein–Barr virus (EBV)-immortalized human B cells [29]. Their level was found to be increased in various diseases [16–19,23]. Whether NaAb can be directly involved in the establishment of a given pathological process has not been established. In the present study we found a high level of IgG autoantibodies directed to a panel of self antigens in the sera of patients with VL contrasting with low level of IgG to TT compared with controls. Patients and controls had been previously vaccinated but not recently boosted with TT. We focused on IgG isotype since it is involved in the memory response after vaccination with TT, and since this isotype shows the most dramatic increase during VL. Although RF could be detected in 54% of VL sera, they did not appear to interfere in the global results of serological tests used in this study. Our results indicate that the polyclonal B cell stimulation, which is common to other parasitic and viral infections, concerned mainly cells secreting polyreactive IgG autoantibodies. In contrast, antigen-driven specific IgG were suppressed during active disease. This 'suppression' is more pronounced in patients with the highest levels of NaAb, in general associated with the presence of RF. This 'transitory immunodeficiency' may be

related to the clinical picture of VL, since it may account for the high susceptibility of VL patients to bacterial infections, as previously reported by Andrade *et al.* [30].

Interestingly, in patients studied before and after recovery we found a decrease of polyreactive antibodies and a spontaneous significant increase of IgG to TT without boosting with TT. This reciprocal imbalance between polyreactive and specific IgG antibodies before and after treatment led us to analyse the B cell subpopulation defined by the expression of the CD5 surface antigen marker. We analysed the PBMC isolated from 16 patients before and after treatment. A significant low percentage of $CD5^+$ per total B cells was found in patients before treatment. This percentage increased significantly in all patients after recovery. This unexpected result is in apparent contradiction to the increased number of B cells expressing the CD5 phenotype reported in rheumatoid arthritis and primary Sjögren's syndrome [31], following allogenic bone marrow transplantation [32], or in patients with infectious mononucleosis [33], all conditions characterized by polyclonal B cell activation and presence of circulating autoantibodies. Our results could be explained by the fact that peripheral blood $CD5^+$ B cells in this specific pathological situation are not representative of the total pool of $CD5^+$ B cells during the course of the disease, and that these cells may be sequestrated in the parasitized lymphoid organs. After recovery these cells could gain the circulation. This hypothesis needs to be validated on analysis of B cell subpopulations in lymph nodes or spleen of patients before and after effective treatment. However, such procedures would be difficult for ethical reasons.

The high level of autoantibodies found in active VL sera was not surprising, since it was reported several years ago [22,23], and explained by non-specific B cell activation. In spite of the increase of total IgG and a correct vaccination against tetanus, the level of IgG to TT was low in patients compared with controls. These abnormalities were reversed by antimonial therapy. Whether they could be the consequence of idiotypic suppression of the specific antibodies by NaAb is presently speculative. It is worth noting that idiotypic suppression of pathogenic antibodies was well documented in both the murine system and humans. For instance, treatment of newborn mice with natural autoantibodies completely suppressed the anti-acetylcholine receptor antibodies when treated mice were later immunized with the acetylcholine receptor [34]. In humans, normal intravenous immunoglobulins are increasingly used in the treatment of human autoimmune diseases to induce idiotypic suppression of pathogenic autoantibodies by polyspecific IgG which are present in large pools of plasma from normal donors [35].

The polyclonal B cell activation, which seems to concern mainly cells secreting natural autoantibodies, may be either due to polyclonal activators, such as parasite-derived mitogens, or the consequence of a particular immune response involved in this parasitic disease. We believe the latter mechanism is more likely. Several studies, reviewed by Sher *et al.* [36], in human VL and murine experimental leishmaniasis have demonstrated that resistance was mediated by Th1 responses, while dissemination was associated with Th2 responses. In active VL, Th2 cells produce IL-4, IL-6 and IL-10, and induce B cell activation and differentiation, which may explain the characteristic hypergammaglobulinaemia found in these patients. Whether these cytokines induce preferentially NaAb by acting on $CD5^+$ B cells is presently unknown. However, several observations have established a link between IL-10 and $CD5^+$ B cells: (i) $CD5^+$ B cells are the main source of B cell-derived IL-10 [37]; (ii) IL-10 is needed for the development of murine $CD5^+$ B cells but not conventional B cells [38]; (iii) a recent study suggested that IL-10 induces activated human B cells to secrete large amounts of IgG, IgA and IgM [39].

Our results, obtained in a situation of a dynamic natural immune response generated by a parasitic disease, support the view that there is a functional dichotomy in the B cell response which possibly could be cross-regulated.

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